

Retinal Degeneration is Rescued in Transgenic rd Mice by Expression of the cGMP Phosphodiesterase β Subunit

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Notes:

Retinal degeneration is rescued in transgenic *rd* mice by expression of the cGMP phosphodiesterase β subunit

(retinitis pigmentosa/photoreceptor/phototransduction)

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ABSTRACT The β subunit of the cGMP phosphodiesterase (PDE) gene has been identified as the candidate gene for retinal degeneration in the *rd* mouse. To study the molecular mechanisms underlying degeneration and the potential for gene repair, we have expressed a functional bovine cGMP PDE β subunit in transgenic *rd* mice. One transgenic mouse line showed complete photoreceptor rescue across the entire span of the retina. A second independently derived line showed partial rescue in which photoreceptors in the superior but not the inferior hemisphere of the retina were rescued. In the latter animals, intermediate stages of degeneration were observed in the transition zone between rescued and diseased photoreceptors. Pathologic changes in the retina ranged from vesiculation of the basalmost outer segment discs in otherwise structurally intact rod cells to photoreceptors with highly disorganized outer segments and intact inner segments. Totally or partially rescued retinas showed a corresponding restoration of cGMP PDE activity, whereas nonrescued retinas had minimal enzyme activity, characteristic of the *rd* phenotype. These transgenic animals provide models for studying the molecular basis of retinal degenerative disease and conclusively demonstrate that the phenotype of *rd* mice is produced by a defect in the β subunit of cGMP PDE.

The retinal degeneration phenotype of the *rd* mouse has served as a model for the study of human retinitis pigmentosa for >30 years (1, 2). The degeneration is inherited in an autosomal recessive fashion and is characterized by a rapid initial loss of rod photoreceptor cells, first detectable by electron microscopy at postnatal day 8, followed by loss of cone photoreceptors (3, 4). By 30 days, a majority of photoreceptor cells have degenerated, producing a severe thinning of the entire retina, which results in apposition of the retinal pigment epithelium with the remaining neurosensory cells (5).

Biochemical studies comparing retinas from normal and *rd* mice have shown elevated cGMP levels in *rd* photoreceptors relative to wild type (6) with a concomitant reduction in cGMP phosphodiesterase (PDE) activity prior to the appearance of structural changes (7). These observations suggested that the defect was in the tetrameric cGMP PDE enzyme, which is composed of two large catalytic subunits, α and β , and two small inhibitory γ subunits (8, 9). Linkage mapping has localized the cGMP PDE β -subunit gene to the same region on mouse chromosome 5 as the *rd* locus (10), whereas the α and γ subunits of cGMP PDE have been mapped to other chromosomal locations (11, 12). These observations correlate with the identification of the β subunit of cGMP PDE as the defective candidate gene in *rd* mice (13, 14). In

addition, several mutations associated with the retinal degeneration phenotype have been identified in the cGMP PDE β -subunit gene (15, 16).

To examine the role of the cGMP PDE β subunit in degeneration and gene repair, we have expressed the complete cDNA of the gene in transgenic mice by using the 5' flanking region of the mouse rod opsin gene to target it specifically to photoreceptor cells. We describe here two lines of transgenic mice that express the cGMP PDE β -subunit fusion gene. One line completely rescues the retinal degeneration phenotype. A second line partially rescues the defect. These results conclusively demonstrate that the cGMP PDE β subunit is the site of the *rd* mutation.

MATERIALS AND METHODS

Transgene Construction. The transgene was made by ligation of the 3.0-kilobase (kb) *Kpn* I/*Xho* I fragment encoding the complete bovine cDNA of the cGMP PDE β subunit (17) to the 4.4-kb *Kpn* I/*Xho* I mouse rod opsin 5' flanking region (18). The 0.6-kb simian virus 40 polyadenylation signal from pGAL5 (18) was ligated to the 3' end of the PDE β -subunit cDNA, producing an 8-kb fusion gene construct (see Fig. 1). All constructs were made in the pBluescript SK(–) vector (Stratagene).

Transgenic Mouse Production and Analysis. Animal usage was in accordance with institutional guidelines. The fusion gene was introduced into fertile one-cell B6D2 F₁ or FVB/n mouse embryos (The Jackson Laboratory). B6D2 F₁ mice have the wild-type +/+ allele, while FVB/n mice are homozygous for the *rd* allele (19). Thus, founder animals derived from B6D2 F₁ mice were bred back into the C57BL/6J *rdle/rdle* background before morphological analysis of retinas. The presence of the homozygous *rd* allele was verified by PCR analysis across an *rd*-specific restriction fragment length polymorphism containing an *Msp* I site. Primers used were 5'-GACAGGCAAACTGAAGAGCT-3' and 5'-GGCTGTTGATCCAAGACCCT-3'.

Southern blot analyses to identify founder animals and to determine transgene copy number were run with 6 μ g of genomic mouse DNA prepared from tail samples (20). DNAs were digested with *Hind*III or *Eco*RI using conditions specified by the enzyme manufacturer (New England Biolabs). Digested DNAs were run on a 0.5% agarose gel (SeaKem ME Agarose, FMC), transferred to nylon membrane (Zetabind, Cuno), and baked dry. Blots were prehybridized in 50% formamide/0.5 M Na₂HPO₄/1 mM EDTA/1% bovine serum albumin/5% SDS (21) and probed with either a rod opsin or a PDE β -subunit cDNA fragment radiolabeled by random priming with [³²P]dATP (22, 23). After overnight hybridiza-

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Abbreviation: PDE, phosphodiesterase.

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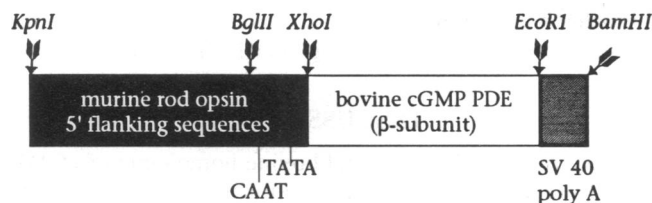


FIG. 1. Fusion gene construct. The 4.4-kb murine rod opsin 5' flanking region was ligated to the complete 3.0-kb bovine cGMP PDE β -subunit cDNA to which the simian virus 40 (SV40) polyadenylation signal was added. The 8.0-kb *Kpn* I/*Bam*HI fragment was microinjected into the male pronucleus of one-cell mouse embryos.

tion, blots were washed at high stringency with $2\times$ SSC ($1\times$ SSC = 0.15 M NaCl/ 0.015 M sodium citrate)/ 0.1% SDS at 65°C and $0.1\times$ SSC/ 0.1% SDS at 65°C for 30 min under each wash condition. Autoradiograms were developed after an overnight exposure to Kodak XAR film. The three oligomers used for PCR analysis for the presence of the transgene were 5'-GAATTCCCAAGAGGACTCTGGG-3', 5'-GACGTTG-GAGAAGGGCACATAA-3', and 5'-CATGATCACAGC-CACGACATC-3', amplifying a transgene fragment or an endogenous rod opsin gene fragment as a control. All animals analyzed were from the first, second, or third generation of breeding from the founder mouse. Retinas from transgenic mice were compared with nontransgenic age-matched siblings ranging from postnatal day 10 to 3.5 months of age.

Tissue Processing of Mouse Eyes for Light and Electron Microscopy. After sacrifice and before enucleation, the orientation of mouse eyes was marked by passing an 8-0 nylon suture through the superior pars plana. Eyes were then opened through the inferior pars plana and fixed in 2% paraformaldehyde/ 2% glutaraldehyde in 10 mM phosphate-buffered saline (pH 7.2). Eyes were further postfixed in 4% OsO_4 , dehydrated in a graded ethanol series, and embedded in Araldite 502 epoxy resin (CIBA Pharmaceutical).

cGMP PDE Assay. Eyes of transgenic and nontransgenic siblings were marked for orientation and enucleated as described above. Each eye was cut into superior and inferior hemispheres. Retinal tissue from each hemisphere was homogenized in a buffer with 10 mM Tris-HCl, pH 7.5/ 1 mM

EDTA. cGMP PDE activity was assayed as described (6). Briefly, the final reaction mixture contained 30 μg of retinal extract, 0.4 mM [^3H]cGMP ($250,000$ cpm), 5 mM MgCl_2 , and 40 mM Tris-HCl (pH 7.5) in a total vol of 100 μl . Tubes were incubated for 15 min at 37°C , and the reaction was stopped by boiling. After cooling, samples were incubated for 10 min at 37°C with 0.3 unit of alkaline phosphatase. This second reaction was stopped by addition of 1 ml of AG1-X2 resin (50 - to 100 -mesh; Cl^- form, diluted $1:3$, Bio-Rad). Tubes were left standing for 30 min and then centrifuged at $3500\times g$. Aliquots of the supernatant were assayed. The percentage of retention of guanosine by the resin was determined spectrophotometrically, and the corresponding factor to correct for nucleoside retention was introduced into the calculations for enzyme activities.

RESULTS

The 8-kb mouse rod opsin/bovine cGMP PDE β -subunit cDNA fusion gene (Fig. 1) was expressed in pigmented C57BL/6J *rdle/rdle* and albino FVB/n mice homozygous for the *rd* allele. Founder transgenic animals were identified by Southern blot and PCR analysis. Two lines of transgenic animals, RP-33 in the FVB/n *rd* mouse line and RP4-28 in the C57BL/6J *rd/rd* genetic background, expressed the transgene. The RP33 transgenic line showed complete rescue of photoreceptor cells (Fig. 2). RP4-28 mice exhibited partial rescue of photoreceptor cells. Both transgenic mouse lines had a single copy integration of the transgene as determined by Southern blot hybridization.

Retinas from transgenic mouse line RP33 and nontransgenic age-matched control siblings were examined at postnatal days 10, 15, 20, and 30 (Fig. 2). No obvious morphological difference between transgenic and nontransgenic retinas was detectable at 10 days of age. However, at 15, 20, and 30 days of age, marked rescue of photoreceptor cells across the entire span of the retina was discernable. Photoreceptor cells from transgenic retinas were normal, whereas age-matched control retinas showed severe loss of photoreceptor inner and outer segments as well as a characteristic thinning of the outer nuclear layer.

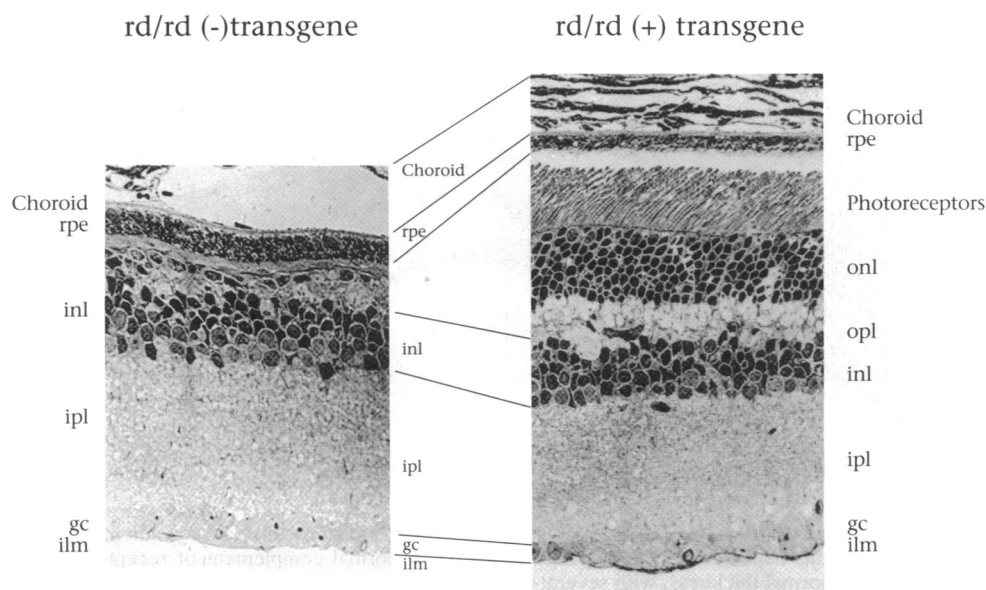


FIG. 2. Light micrographs of retinas from RP33 siblings negative and positive for the PDE β -subunit transgene at postnatal day 30. In the retina negative for the transgene, photoreceptors have degenerated, such that the inner nuclear layer (inl) is directly apposed to the retinal pigment epithelium (rpe). The transgenic retina appears normal, exhibiting complete outer plexiform (opl) and outer nuclear (onl) layers. A full complement of rod and cone photoreceptors possessing full-length outer segments is present. ipl, Inner plexiform layer; gc, ganglion cells; ilm, inner limiting membrane. ($\times 225$).

Retinas from transgenic mouse line RP4-28 and control siblings were examined morphologically at postnatal days 10, 15, 30, and 60 and at 3.5 months of age. Like the RP33 transgenic line, no obvious morphological difference was detectable at 10 days of age. However, at postnatal days 15, 30, and 60 and at 3.5 months, a superior temporal to inferior nasal gradient of rescue was observed (Fig. 3*a*). Photoreceptors in the superior temporal region of the retina retained a normal morphology, while photoreceptors in the inferior nasal region had completely degenerated. In the central retina, degeneration was intermediate in severity. This region retained a full complement of photoreceptor nuclei and inner segments, but most outer segments were highly disorganized or absent. At the transition from superior to central retina, rods were structurally intact, with the exception of abnormal vesiculation of the basalmost disc membranes of the outer segment (Fig. 4).

To correlate enzyme expression with photoreceptor rescue, levels of cGMP PDE activity were compared in RP4-28 and RP33 transgenic and nontransgenic age-matched siblings. Retinas were bisected into superior and inferior hemispheres and each hemisphere was assayed for cGMP PDE activity (Fig. 5). Retinal samples from nontransgenic *rd* siblings showed minimal or no activity. cGMP PDE activity in the superior and inferior retinal hemispheres of one RP33 animal analyzed, approached that observed in wild-type *+/+* animals. In two RP4-28 transgenic animals examined, the level of enzyme activity in the superior hemispheres was higher than in the inferior hemispheres. The lower cGMP PDE activity of the inferior hemispheres correlates with reduced numbers of rescued photoreceptors. These results

show that expression of the rod opsin/cGMP PDE β -subunit transgene is capable of restoring cGMP PDE activity.

DISCUSSION

We have expressed the normal bovine homologue of cGMP PDE β subunit, the candidate gene for mouse retinal degeneration, in transgenic mice homozygous for the endogenous *rd* allele. The RP33 line of transgenic animals showed photoreceptor rescue across the entire span of the retina (Fig. 2). The RP4-28 line showed a superior to inferior gradient of rescue (Fig. 3).

Both uniform and gradients of expression have been observed in other transgenic mouse lines using the rod opsin promoter (18, 24). The gradient of rescue in the RP4-28 transgenic mouse line (Fig. 3*a*) mirrors spatial expression patterns previously reported in one transgenic mouse line generated by using the mouse rod opsin promoter and the *Escherichia coli* β -galactosidase reporter gene (18) (Fig. 3*b*). Another transgenic line expressing the rod opsin/ β -galactosidase transgene showed uniform β -galactosidase activity across the retina (18), similar to the complete rescue of photoreceptor cells observed in the RP33 mouse line.

The spatial pattern of expression in the RP4-28 transgenic mouse line appears to be dependent on upstream 5' flanking sequences of the rod opsin gene. This pattern of expression has been observed not only in constructs using β -galactosidase (18) but also with rod and cone transducin structural genes (J.L., M.I.S., C. Raport, and J. Hurley, unpublished data). It is plausible that lower levels of expression lead to intermediate levels of degeneration, including vesiculation

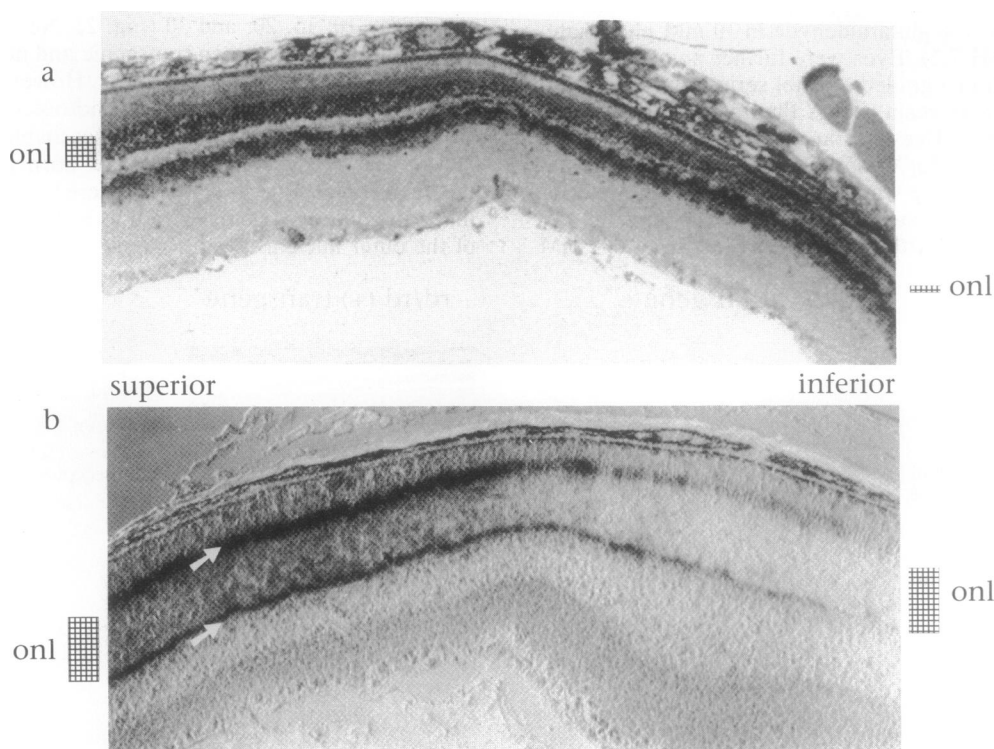


FIG. 3. Light micrographs of transgenic retinas. (a) Thirty-day-old RP4-28 transgenic mouse displays a gradient of photoreceptor rescue from the superior (left) to the inferior (right) hemisphere. The superior retina has a normal complement of receptors with intact structure and well-ordered outer segments. It is of normal thickness, with several rows of receptor nuclei comprising the outer nuclear layer (onl) (grid). The rescue effect diminishes toward the inferior retina, resulting in a progressive loss of rods until a single row of cone nuclei remains (single boxes). The central retina retains many receptors with intact inner segments but disorganized outer segments. ($\times 110$.) (b) A superior to inferior gradient of expression was also seen in an independently derived, nondegenerate line of transgenic animals expressing a rod opsin/ β -galactosidase fusion gene (18). The onl is of uniform thickness from superior to inferior (grids). The β -galactosidase reaction product (arrows) appears as two dark bands sandwiching the onl caused by accumulation of reaction product in the inner segments and synaptic terminals of the rods. Staining is most prominent in the superior aspect and diminishes in the inferior aspect of the retina. ($\times 225$.)

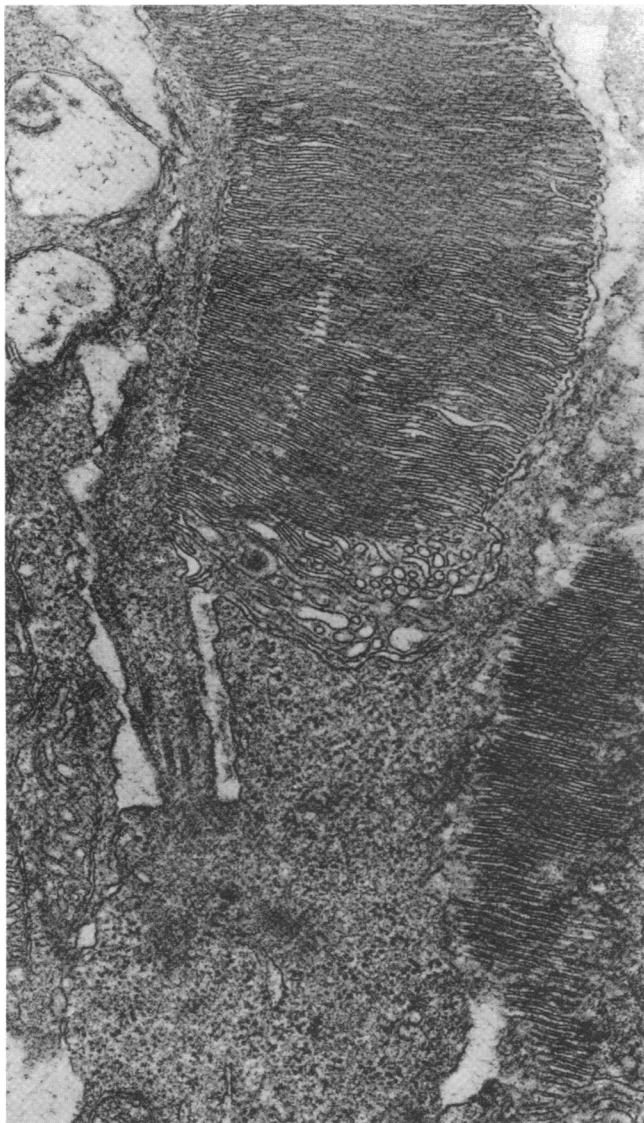


FIG. 4. Electron micrograph of a rod from the central retina of a 30-day-old transgenic RP4-28 mouse. In this region of the retina, the rods display vesiculated discs in the basal region of the outer segment. Note the morphologically normal disc membranes above this region synthesized just a few days earlier. ($\times 37,800$.)

observed at the base of otherwise structurally normal rod outer segments. The presence of a large stack of intact disc membranes in the outer segment above this vesiculation suggests that the gradient of photoreceptor rescue may be the result of a change in transgene expression. Abnormal disc morphogenesis in the most recently synthesized discs suggests that a decrease or shutdown of cGMP PDE β subunit may ultimately affect disc membrane formation. Alternatively, an abnormal ratio of the transgene product relative to other disc proteins may lead to vesiculation. Vesiculation was not observed in retinas of RP33 transgenic animals nor has it been reported in *rd* mice.

The asymmetry of rod opsin-driven transgene activity in this animal model may extend its usefulness as a model of retinitis pigmentosa in humans. Aberrant vesiculation has previously been reported in a patient with retinitis pigmentosa (25). In addition, recent reports of retinitis pigmentosa in humans with the proline to histidine mutation in codon 23 of the rhodopsin gene have described a "sectoral" distribution of degeneration, in which the inferior hemisphere was the most severely affected (26, 27).

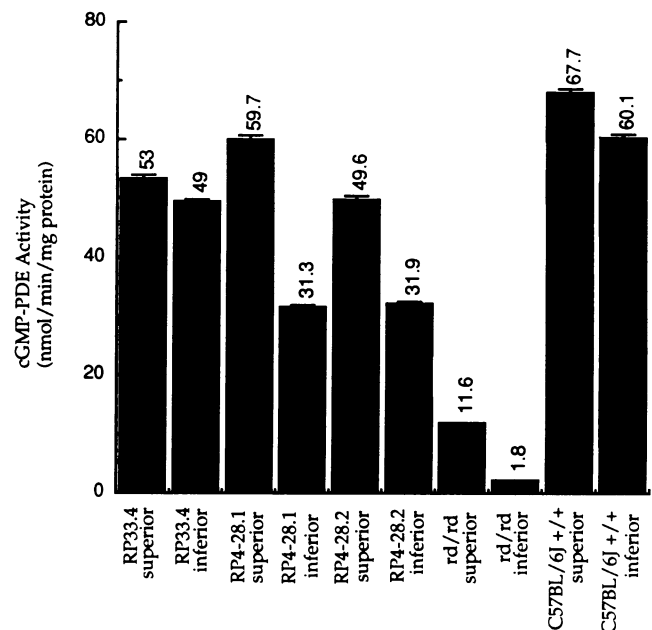


FIG. 5. Histogram of cGMP PDE activities in 30-day-old transgenic vs. control *rd/rd* mice. cGMP PDE activity in the superior and inferior retina hemispheres of completely rescued RP33 transgenic mice approached levels observed in the C57BL/6J +/+ retinas. PDE activity in retinas from two RP4-28 mice was examined. Lower levels of activity in the inferior hemispheres correlated with fewer rescued photoreceptors. Bars represent cGMP activities in individual retinal samples obtained from the superior or inferior hemiretina based on four determinations per sample. Error bars are 1 SD of the mean.

In summary, our results indicate that the expression of a normal cGMP PDE β subunit is sufficient to restore cGMP PDE activity and rescue photoreceptor cell degeneration in both pigmented C57BL/6J and albino FVB/n *rd* mice. These observations support the conclusion that the retinal degeneration phenotype arises from a defect in the cGMP PDE β -subunit gene.

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